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IN VITRO STUDIES OF SEX STEROID EFFECTS
ON HUMAN MAMMARY CARCINOMA
AND EXPERIMENTAL RAT MAMMARY TUMOUR

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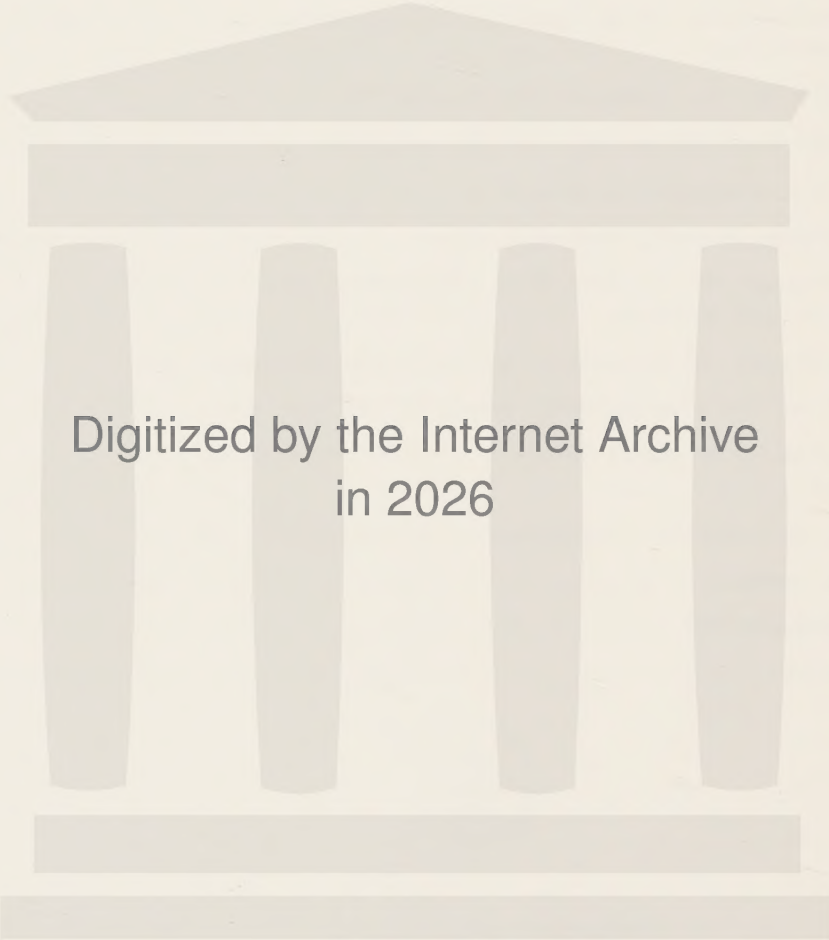
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serum is added, several waves of DNA synthesis occur. The factor in serum that induces mammary epithelial proliferation — mammary growth factor, MGF — has been partly purified by Turkington (43) from human serum and is described as a polypeptide of low molecular weight. A synthetic serum substitute was described by Lasfargues et al. (24) which maintained neoplastic but not normal mammary tissues in vitro.

The mammary neoplastic cells might or might not retain the property of specific cell regulation by hormones. For example, the rate of DNA synthesis of the C₃H mouse mammary tumour is influenced by insulin. This also applies to normal mammary gland of the Fisher rat, but not to the R 3230 AC Fisher rat mammary tumour (42). The concept of retained specific cell regulation by hormones in neoplastic tissue, derived from hormone target tissue, forms the biological basis of hormone therapy of cancer.

Hormone therapy in human mammary carcinomas is today given mainly in widespread disease, either as hormone substitution by steroids or as endocrine ablative operations (oophorectomy, adrenalectomy, and hypophysectomy). Hayward (15) and Stoll (39) extensively review the subject. Hormone responsiveness varies widely between different types of tumour, e.g., endometrial and mammary carcinoma, where the former responds more uniformly than the latter to the treatment. But it also varies between different tumours of the same type. Thus, it is depressing that only about one in three of patients with metastasizing mammary carcinoma benefit by hormone therapy. Many patients are given useless treatment with many unwanted side-effects while other palliative treatments are delayed. However, one in three respond well, and among these, a few respond excellently and remissions of long durations — for up to twenty years — have been recorded. One of the great problems of hormonal therapy is prediction of response, i.e. previous selection of patients that will or will not respond to the treatment.

Prediction of hormonal responsiveness of human mammary carcinoma

At present, there is apparently no reliable method for predicting hormonal response of a given tumour in a patient. In clinical practice, we are confined to such criteria as menopausal state, span of time between primary operation and relapse, response of previous hormone therapy, or similar methods (15). The measurement of the excretion of urinary steroids does not seem to have conferred greater accuracy than clinical criteria (15). The unreliability of clinical criteria for the prediction of individual outcome of endocrine therapy made it desirable to attempt to develop in vitro methods that could assess the endocrine sensitivity of a given tumour before beginning the actual therapy. Many different approaches have been tried during the years to find such methods and much of the literature on the subject is reviewed by Hilf (17) and Matthias (28). At the present time, interest has focused mainly on three different principles: a) to study the capacity of specific binding of steroids to the tumour tissue; b) to study the effects of steroids on certain aspects of cell metabolism; c) to study the influence of steroids on tumour cell proliferation.

Selective uptake of oestrogen into human mammary carcinomas in vivo was first demonstrated by Folca et al. (9) and a correlation between this parameter and subsequent clinical course was suggested. Later, it was independently demonstrated with in vitro methods that about 50 per cent of mammary carcinomas in an unselected material specifically bind 17- β -oestradiol (12, 14, 19, 20, 27). Receptor binding of oestradiol and subsequent outfall of endocrine therapy (adrenalectomy, hypophysectomy, oophorectomy, or administration of adrogen or oestrogen) have been compared by Jensen et al. (19) in 42 patients. Of 13 patients with specific binding of oestradiol

to their tumours, 10 responded to hormone therapy. Of 26 patients with no specific binding, 1 responded to hormone therapy and 25 did not; 3 borderline binders did not respond.

It is now established that mammary carcinomas metabolize steroids. The subject is reviewed by Adams and Wong (1), Griffiths et al. (13), Dao and Libby (5). An important step in steroid metabolism is sulphurylation of the molecule. Dao and Libby (5) studied this process in 109 patients with disseminated mammary carcinoma. Of the tumours with low sulphokinase activity, 15 per cent responded to adrenalectomy. Of the tumours with high sulphokinase activity, 73 per cent responded. However, Leung et al. (26) could not establish a definite correlation between sulphurylation activity and clinical response to adrenalectomy in 22 similar patients. Braunsberg et al. (2) investigated the correlation of specific binding of 17- β -oestradiol and sulphurylation of the hormone in vitro of tumours from 35 patients and found no such correlation.

The research group around Salih, Flax and Hobbs, using organ culture technique, studied pentose shunt activity, i.e. anaerobic glucose metabolism. They worked with a defined medium and found dependence on steroids, prolactin, and growth hormone for maintenance of the pentose-shunt activity in some tumours (6, 33, 34, 36). Of 130 tumours, 14 were thus testosterone dependent. Of the patients in this in vitro dependent group, 8 were treated with "anti-androgen measures", i.e. oestrogen administration, adrenalectomy, or hypophysectomy; 6 of them responded to the treatment. The validity of this correlation was criticized by Heuson (16). Savlov et al. (35) studied dehydrogenase activities of 130 mammary carcinomas at the time of operation and found levels in carcinomas higher than in fibrocystic disease or normal mammary gland, but concluded that certain prediction was impossible from their material.

There are some investigations of morphological changes in organ cultures of human mammary carcinomas. Two recent ones are those by Wellings and Jentoft (46) and Lagios (23). However, this parameter of growth has many limitations, e.g., heterogeneity of carcinoma tissue, and difficulties of grading necrosis and proliferation. Stoll (38) considered the matter and stated: "Morphological evidence of growth inhibition in organ culture is probably the least suitable parameter in the case of breast cancer".

The study of DNA synthesis in tumour tissue could be a more objective parameter of tumour growth. Turkington, using autoradiographic technique, studied the influence of insulin on mouse and rat mammary tumours (42). He found that insulin mediated changes in DNA synthesis, mirrored by an increase in the number of cells synthesizing DNA rather than in an increased rate of DNA replication per cell. He concluded: "the regulation of the initiation of DNA synthesis represents the most significant control mechanism in determining the rate of cell proliferation".

Such studies have been performed by Burstein et al. (3) who used cell suspensions, and by Riley et al. (32) who used monolayer cultures. The cells were incubated with hormones for 48 hours and the influence on the incorporation of tritiated thymidine into DNA was studied. However, heavy selection of cells occur in these long term suspensions or monolayers.

Aim of the present investigation

Based on the above considerations, the initial aim of the present study was to investigate hormone effects on human mammary carcinoma in vitro, measured as rate of DNA synthesis, and to study whether these effects were correlated to the clinical course of the patient.

The secondary aim was to investigate whether the carcinogen induced rat mammary tumour showed the same characteristics as human mammary carcinoma concerning hormone responsiveness in vitro; this with correlation of hormonal response in vivo-in vitro in mind. During the course of the work, hormone effects in vitro were found in tumours not generally considered hormone responsive. The thesis has then centered around the question of specificity of sex steroid effects when studied in vitro with the methods used.

The studies are, or will be, published in five papers which form the basis of this thesis. They are referred to in the text by their corresponding Roman numerals.

- I. Human mammary carcinoma studied for hormone responsiveness in short term incubations. Together with L. Håkansson.
Acta Chir Scand 140:95-99, 1974.
- II. 7,12-DMBA-induced rat mammary tumour studied for hormonal responsiveness in vitro. I. Short term incubation of cell suspensions.
Submitted for publication in Acta Path Scand, Sect A.
- III. 7,12-DMBA-induced rat mammary tumour studied for hormonal responsiveness in vitro. II. Organ cultures.
Submitted for publication in Acta Path Scand, Sect A.
- IV. Growth quantitation of human mammary cancer in organ tissue culture. Together with H. Danielsson.
Am J Surg July 1974, in press.
- V. Hormone effects on human mammary cancer in organ cultures.
Submitted for publication in Am J Surg.

TECHNICAL CONSIDERATIONS OF THE METHODS

Two in vitro culture methods were used in the present work. One was a cell suspension technique, originally described by Nordqvist (31). Here, epithelial cells are suspended in the culture medium during the entire incubation (papers I and II). The other was an organ culture method where small explants are cultured on rafts in a gas-liquid interphase. Only for the last four hours are the explants submerged. This was to make the incorporation of labelled thymidine (H^3 -TdR) as large as possible, as described in paper IV. The main advantage of the organ culture methods is — apart from greater oxygen availability to the tissue — that epithelial cells and stroma cells are in contact with each other during the culture procedure. The organ culture method also permits longer duration of hormone application which could be of importance for the realization of effects which cannot be observed in the short-term incubation method.

Cell suspension method

The epithelial cells of human mammary carcinoma are less adherent than the connective tissue of the tumour. Thus, by merely cutting into the tumour, epithelial cells spill out into surrounding medium, as originally described by Lasfargues and Ozello (25). This technique was used for preparation of the cell suspensions in the present investigations. However, the knife-cutting technique was abandoned in favour of mincing with sharp scissors, which worked equally well and had the advantage of being considerably faster. The cell suspensions from human mammary carcinomas were well

dispersed, i.e. cells were mostly single with a few clusters of up to about ten cells intermingled. With the rat DMBA tumour, however, the cells adhered much more, and the suspensions were mostly made up of small clusters of cells, each consisting of up to twenty cells. This was also the case with the sarcomas: human sarcoma cells detached more easily than rat sarcomas. The number of cells in suspension was difficult to assess; cell counting was therefore abandoned in favour of reliance on the DNA content as an index of cell number. The DNA contents in each tube differed in different experiments, but was relatively constant within experiments. DNA was used for relating cpm to cell number of a tube. The ratio cpm/DNA thus expresses newly synthesized DNA per cell. It was given in arbitrary units as a-values, as described in paper I.

To control whether the inequality of DNA contents between experiments had any influence on the registered hormone effects, DNA-values were plotted against hormone effects. No correlation between these parameters was found, which indicates that the number of cells in suspension did not influence the magnitude of response, i.e. difference in H^3 -TdR incorporation between control tubes and hormone treated tubes.

The viability of the cells was controlled in a few instances by dye exclusion test. However, this test proved difficult to quantitate, and the ability of the cells to synthesize DNA was taken as a more reliable index of cell viability. Four rat tumours were also tested for RNA synthesis; Fig. 1 in paper II gives the result.

As that figure shows, total DNA of the suspensions decreases to about 60 per cent of the original value during five hours of incubation, and a decrease is also evident after three hours in three of the four studied suspensions. This decrease probably reflects mechanical damage to the cells during the preparation.

However, DNA and RNA synthesis remain fairly steady, indicating that the damaged cells do not participate in nucleic acid synthesis.

The resultant a-values decline little; the decline is more evident for RNA synthesis than for DNA synthesis.

The main advantage of the cell suspension method is that the entire tumour material is minced; thus the risk for selection of special clones is minimized. However, the initial cell loss can give rise to cell selection.

Organ culture method

Here, small pieces of tumour are cultured in a liquid-gas interphase, except for the last four hours when the explants are submerged in the medium. The main disadvantage of the method is that media penetrate only to about 150 microns from the surface of the explants, and a central necrosis almost always occurs. This is seen in Fig. 1 of paper III and Fig. 4 A of paper IV.

The necrosis could influence the labelling of the tissue with H^3 -TdR, as the radioactive nucleotide might have to compete with cold thymidine emanating from degraded DNA. If so, it should be of the same magnitude for controls as for hormone treated cultures and should not influence the registered hormone effects. As the incorporation curves for control flasks show (papers III, IV and V), cells in the explants in most instances actively multiply after the first 24 hours. (A notable exception is sarcoma No. S2, paper V, where DNA synthesis declines during the first 48 hours and then increases.) When slides were histologically examined, this growth was confirmed, but no attempts were made to quantitate by counts of labelling index or mitotic index.

Comparison of the cell suspension and the organ culture methods

Stimulations of DNA synthesis were seldom recorded in cell suspensions (one of 128 determinations of human mammary carcinomas; nine of 310 determinations of rat carcinomas or sarcomas) and some of these may be random. To investigate whether this was due to shortcomings in the cell suspension technique, the following experiment was made.

Human mammary carcinomas were tested simultaneously with hormones during four hours' incubation, as described in papers I and V. Testosterone, progesterone, and $17\text{-}\beta\text{-oestradiol}$ were tested at concentrations shown in Fig. 1. The sole difference between the different parts of the experiment was that both cell suspension and tumour pieces were used and could thus be compared. Fig. 1 shows the effect curves (mean difference between three control tubes and two hormone tubes). In order to make comparisons possible, $\log_{10}$ cpm was used for both suspensions and pieces. Significance of hormone effect was tested with F-test, as described in paper I, and limits of significance are given graphically. One tumour yielded enough material to be tested at all concentrations of the hormones. It is represented by the right inserts of Fig. 1 A-C. The left inserts of Fig. 1 A-C represent three individual tumours. With testosterone (Fig. 1 A), both tumours were significantly stimulated at either 0.5 or 5 $\mu\text{g/ml}$ when incubated as pieces, but were not significantly affected when incubated as suspensions. At 50 $\mu\text{g/ml}$, one tumour (left insert) was significantly inhibited with both methods. The other tumour was significantly inhibited as suspension, but not as pieces, although a tendency towards inhibition is evident.

With progesterone, the two methods gave uniform results (Fig. 1 B). One tumour was not affected by the hormone, the other was inhibited at 80 µg/ml.

With 17-β-oestradiol, one tumour (Fig. 1 C, left insert) was stimulated at 0.2 µg/ml when tested as pieces, but not affected as suspension. Other concentrations did not significantly affect the tumour. The other tumour (right insert) was not significantly affected at any concentration with either method, but when tested as pieces, there was a tendency towards stimulation at the lower concentrations; this was not present in the suspensions.

It is concluded from these experiments that the stimulatory capacity of the hormones is more often overlooked with short-term incubations of cell suspensions than with tissue pieces. One possible explanation is greater mechanical damage to cells when a suspension is prepared; another, that intact relation between stroma cells and epithelial cells is important for cell growth in vitro.

Effects of culture time in organ cultures

Hormonal effects in a tumour varied greatly when studied at different length of time of organ culture (papers III and V). This variation can be the result of the continuous hormonal treatment. But it was also thought that the artificial conditions in vitro might change the hormonal responsiveness of a tumour. Therefore, the following experiment was performed.

Three individual mammary carcinomas were cultured with testosterone, progesterone, and 17-β-oestradiol, respectively, at standard concentrations and culture technique (paper V). Three series were set up from each tumour.

The first was cultured for 4 hours with hormone added.
The second was cultured for 72 hours with hormone added.
The third was cultured for 68 hours with control medium and for 4 additional hours with hormone added, making the total incubation time 72 hours.

Thus, the only difference between the two latter series was in duration of hormone treatment — 72 versus 4 hours. The only difference between the first and the last series was in length of culture time: 4 y. 72 hours. Fig. 2 records the results.

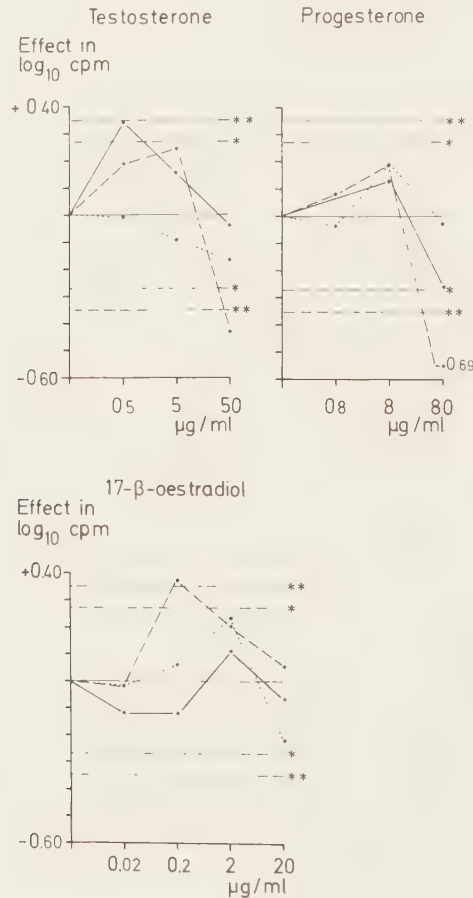


Figure 2. Comparison of changes in hormone response due to length of culture time. Each insert represents an individual human carcinoma cultured for 4 or 72 hours. Dose-response curves are given with significance limits of F-test between "effect" and "general technical error".

(—) is 4 hours incubation with hormone. (---) is 72 hours with hormone. (.....) is 68 hours without and final 4 hours with hormone added.

With progesterone and 17- β -oestradiol, the results are clear-cut: there is no significant effect of mere culture time; the effect curves for cultures treated for 4 hours are almost similar irrespective of total culture time. Progesterone treatment for 72 hours made the cultures more sensitive to the highest concentration, 80 $\mu\text{g/ml}$, which was inhibitory. 17- β -oestradiol significantly stimulated at 0.2 $\mu\text{g/ml}$ after 72 hours of continuous treatment. With testosterone, the findings were more complicated. After four hours of incubation with hormone, 0.5 $\mu\text{g/ml}$ significantly stimulated. Incubation for 68 hours obviated this potency for testosterone stimulation. Hormone treatment for 72 hours made the culture sensitive to the highest concentration, 50 $\mu\text{g/ml}$, which significantly inhibited.

These experiments suggested that the culture procedure per se can influence the tumour tissue that the capacity to accelerate growth by hormonal treatment can diminish.

ASPECTS ON THE STATISTICAL ANALYSIS OF THE DATA

The use of Fisher's method of variance analysis makes it possible to separate different sources of variation in a material and to estimate their size. This method presumes a reasonably normal distribution of the data. In biological work, data often do not distribute normally. An example of this is the cpm/DNA ratios when the cell suspension method is used. Such ratios are not normally distributed, but the distribution can be transformed to near-normality by logarithmation, as demonstrated by Nordqvist (31). As paper III shows, cpm data registered when the organ culture method was used deviated slightly but not significantly from normality; the same applied also for $\log_{10}$ cpm values. As the latter data are simpler to analyse statistically, they were preferred.

The basic phenomenon to be studied is the possible change in DNA synthesis caused by the addition of certain hormones to the culture media. Such an effect is reasonably of a multiplicative nature — e.g., a certain hormone can at a certain concentration double the DNA synthesis. When log data are used, a multiplicative effect is transformed to an additive effect, which makes statistical analysis easier. The significance of recorded differences between two experimental groups — e.g., control cultures and hormone cultures — must be evaluated by comparison with the variability present between identically performed cultures; i.e., the "technical error" variance. There are many causes of the latter variance, e.g., variations in cell number between different tubes in the cell suspension method, and variations in explant size in the organ culture method, pipetting errors, etc. The size of the technical error variance can be estimated from a number of double or triple determinations performed in "identically" prepared cultures. A few double or triple determinations could scatter more than is expected from the error variance estimated from the other replicate determinations within a certain series. Bartlett's test was applied to all materials in order to identify such deviating determinations. Their cause may be technical accidents in one of the cultures included, usually in the form of a substantial loss of radioactivity in one of the replicate cultures. The deviating determinations were excluded from the estimate of the general error variance. Studies of possible hormonal effects based on such determinations were made with the aid of a correction devised by Welch (45). The general technical error variance, determined as described above, was remarkably constant for a given method and tumour type. The experiments described in paper IV were made about eight months before the experiments described in paper V; none the less, the estimates of the technical error variances were almost identical; 0.023 at 175 d.f. and 0.022 at 379 d.f., resp.

Analysed thus, certain tumours showed marked reactions to added hormones, others showed no, or only faint, reactions. In order to ascertain that such variations are not random, the statistical interaction between the two sources of variation: "between tumours" and "between control and hormones" (i.e., the actual hormone effects studied), was analysed. When such interaction was statistically significant, this was taken as evidence that the tumours had indeed reacted differently. That difference could, in one or other way, be due to tumour individuality, but it could also be due to variations obtained on different experimental days, as the different tumours were tested in different experiments. The presence of such an interexperimental technical variation was looked for in specially designed experiments (papers II and III). One and the same tumour was then run in a number of parallel experiments so designed that all reasonable interexperimental variables were included (different media, different alcohol solutions, different batches of glass-ware, etc.). With the cell suspension method (paper II), no significant interexperimental variation was found. This was indirectly supported by the findings with cholesterol instead of sex steroids added (paper II) — no variability in response between different tumours being found. On the other hand, with the organ culture method, a significant interexperimental variation could be demonstrated (paper III). Although small, this source of variation thus enters the variation in response registered between different tumours, but it was also shown in that paper that the interexperimental technical variation could not explain all intertumour variability.

The demonstrated intertumour variability is important from the standpoint of specificity of the hormone effects, especially when inhibiting effects were recorded. If these were unspecific because of a toxic action of the steroid molecules, it would be reasonable to suppose that the toxic action should be relatively uniform, and that all tumours should react in a relatively similar way. The

presence of intertumour variability fits the explanation — supported also by other findings — that differences exist between the tumours and are of importance for the in vitro reactions to hormones in the systems used. Naturally, this does not necessarily mean that such differences are biologically meaningful in vivo or can be used for prediction of responses to endocrine therapy.

The results presented in paper I on human mammary carcinoma and 17- β -oestradiol at 20 μ g/ml can be so interpreted that the tumours can be divided into two groups: one responsive to the hormone and the other non-responsive; within each group, the intertumour variability was negligible. The reacting tumours made up approx. half of the total material (13/28 tumours) — this agrees well with the percentage of human mammary carcinomas that show oestradiol receptor activity in vitro (12, 14, 20, 27).

In the suspension experiments, culture time was maintained at 3 hours. In the organ culture experiments, culture time varied between 4 and 72 hours. The original idea was to find the optimum culture time when DNA synthesis was maximum and hormone effects could be most clear-cut. But it was soon found that the reaction of a specific tumour varied at different culture times, not only in degree of reaction, but also in type of response: at one culture time a significant stimulation of DNA synthesis could be found; at another, a marked inhibition. The differences were not haphazard; they formed definite "time-response"-curves, as papers III and V show, and the different tumours varied in this respect, as seen from the secondary interaction variance between the three sources of variation: "between tumours", "between culture times", and "between controls and hormone-treated cultures".

The effect of culture time might be due to two different mechanisms: the different duration of action of the hormone; or changes occurring in the cellular composition of the cultured pieces. One

component in the first possibility which cannot be completely excluded is that steroid metabolism occurs in the cultures. A possible way in which duration of culture per se could influence the result of hormone addition is that, in the explanted pieces of tumour, a polyclonality exists with respect to hormone reactivity. Different kinetics of the different clones, with respect to cell proliferation and/or cell death in culture, could result in different overall effects of added hormones in a way similar to that discussed by Trop   (44) in his in vitro analysis of growing polyclonal mouse sarcoma.

COMPARISON OF HORMONE EFFECTS IN MAMMARY TUMOURS AND SARCOMAS

The two types of tumours and the two culture methods were compared by recording the number of stimulations and inhibitions for each separate material, disregarding type of hormone, hormone concentration, and culture time. Table I gives the numbers together with numbers of tumours. Fig. 3 graphically records the percentages of responses.

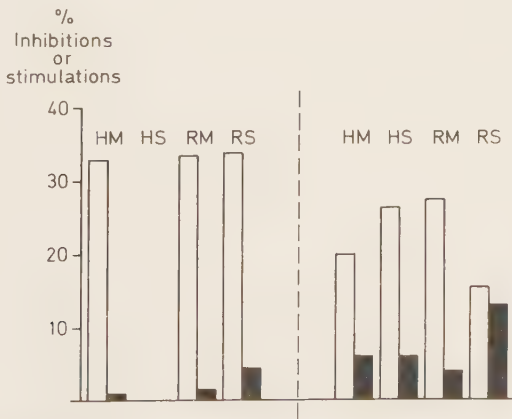


Figure 3. Percentages of recorded stimulations (black staples) and inhibitions (white staples) of total number of recorded effects disregarding hormone concentration, type of hormone, and culture time. Left half represents cell suspensions; right half, organ cultures. HM = human mammary carcinomas, HS = human sarcomas, RM = rat mammary tumours, and RS = rat sarcomas.

Table I

Method and type of tumour	No. of recorded stimulations	No. of recorded inhibitions	Total No. of recordings	No. of tumours with stimulations	No. of tumours with inhibitions	Total No. of tumours
Cell suspension of human mammary carcinoma	1	42	127	1	22	28
Cell suspension of human sarcoma	0	0	20	0	0	2
Cell suspension of rat mammary tumour	2	50	150	2	15	15
Cell suspension of rat sarcomas	7	54	160	2	16	16
Organ culture of human mammary carcinoma	8	27	136	5	8	10
Organ culture of human sarcoma	7	32	120	3	4	4
Organ culture of rat mammary tumour	6	41	150	2	14	15
Organ culture of rat sarcomas	23	28	180	6	13	16

Table I. Summary of recorded stimulations and inhibitions of different tumour types and culture methods, disregarding hormone concentration and type, and culture time. Left half of the table gives number of recordings; right half, number of tumours.

With cell suspensions, the percentage of inhibitions are remarkably similar (around 33 percent) in human mammary carcinomas, rat mammary tumours, and rat sarcomas. When the stimulations are considered, only one is recorded in the human mammary carcinoma material; this could be random, as discussed in paper I. For the rat mammary tumours and sarcomas, the percentages are also similar and are due to effects in two tumours of each type (Table I).

Human mammary carcinomas and sarcomas studied as organ cultures also react very similarly with respect to the number of stimulations and inhibitions. The situation is somewhat different for rat mammary tumours and sarcomas in organ culture, where many more sarcomas are stimulated than are the mammary tumours. Also, the latter tumour type is more often inhibited than the sarcomas. Fig. 3 also provides additional information on the difference between the cell suspension and the organ culture method, although it must be remembered that the considerations below apply to different tumours, different hormones, and different culture times. With these limitations, the following observations can be made: There are generally more stimulations recorded with the organ culture method. This comparison, although less specific than the experiment described in Chapter 3, confirms the conclusion that stimulations are less often seen with the cell suspension method than with the organ culture method. The percentage of recorded inhibitions also differs in that they are more often seen with cell suspensions.

When two human sarcomas were tested as suspensions (paper I), no significant effects were seen. Fig. 4 shows the dose-response curves of these sarcomas. In the light of what is said above and in Chapter 3, this absence of effects might be due to the technique used, i.e. effects might have been underestimated, although a genuine resistance to hormonal treatment cannot be excluded.

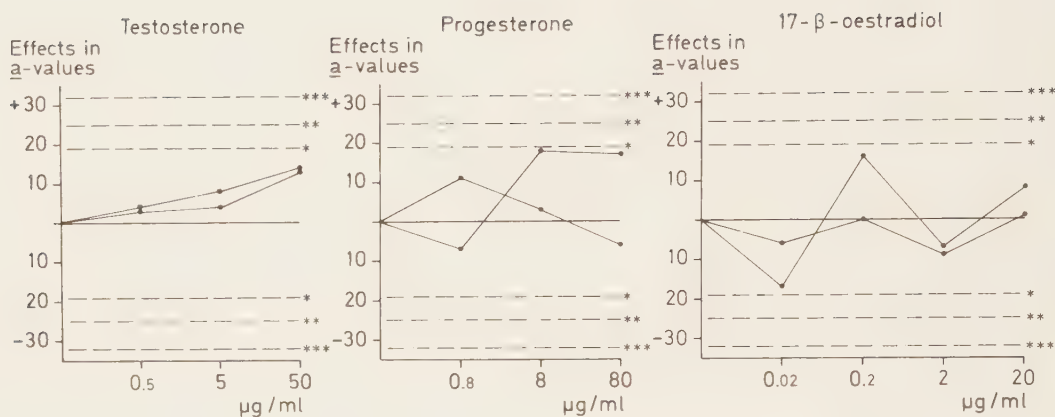


Figure 4. Two human sarcomas tested as cell suspensions for 3 hours. Dose-response curves are given. Dashed lines give significance of F-test between "effect" and "general technical error".

More human sarcomas must be tested as cell suspensions before this question can be answered with certainty.

To summarize: Hormone effects were found with both sarcomas and mammary tumours of the two species investigated. These effects cannot be explained as merely technical or unspecific toxic effects. The cell suspension method, compared with the organ culture method, tends to underestimate possible stimulations and overestimate the number of inhibitions.

GENERAL DISCUSSION, HYPOTHESES, AND SPECULATIONS

Many possible methods are available for studying the influence of sex steroid hormones on tumour tissue under in vitro conditions. Some of these are briefly outlined in the Introduction. The value of such tests for the prediction of the in vivo response of tumours

to endocrine therapy is difficult to assess. One possibility is to search for a correlation between the in vitro response and the in vivo reactivity. This can fairly easily be done with experimental tumour material, but such studies are tedious on human material, partly because of the difficulties of evaluating tumour regression or progression in a specific patient.

In the present thesis, the author has tried to evaluate two in vitro methods, both registering the effect of added sex steroids on DNA synthesis. Definite and variable effects were registered with both methods. As a first effort to investigate whether the registered effects were indeed specific in the sense that they were obtained only with tumours which are, in vivo, susceptible to hormones, biological controls were run with human sarcomas or rat sarcomas, either induced chemically or with virus. The main, and unexpected, finding was that not only mammary tumours, but also sarcomas, showed in vitro susceptibility to the studied sex steroids and that no marked quantitative or qualitative difference in response existed.

There are many alternative explanations for this finding; some are discussed here. The simplest is that the registered "effects" are random variations caused by uncontrolled experimental variables. The general way to evaluate the effects has been to compare them — by variance analysis or t-tests — with the so-called "general error variance", i.e. the variation demonstrable between replicate cultures. Other technical variations might be present which are not included in that calculation, as discussed above at some length. Despite a careful search for such uncontrolled variables, no explanation was found for the registered "effects". Possibly, single "effects" are artefactual — e.g., due to a toxic impurity in one of the stock solutions used — but this must be rare. This analysis also showed that the variability in response recorded between different tumours in both methods could not be explained

by an interexperimental technical variation, but that the response of each tumour is, in one or other way, due to individuality of that tumour.

Another obvious explanation for the similarity of response between mammary tumours and sarcomas is that the high concentrations of sex steroids used markedly differ from the "physiological" concentrations present in the circulation. Fotherby and James (11) give the following ranges of the three main sex steroids in women during the menstrual cycle: testosterone 5×10^{-2} to 10^{-1} $\mu\text{g/ml}$; progesterone 10^{-3} to 2×10^{-2} $\mu\text{g/ml}$; oestrogens 1×10^{-4} to 5×10^{-4} $\mu\text{g/ml}$. Postmenopausal women had oestrogens at a concentration of 5×10^{-5} $\mu\text{g/ml}$. These values should be compared with those used in this study - testosterone 0.5 to 50 $\mu\text{g/ml}$, progesterone 0.8 to 80 $\mu\text{g/ml}$, oestradiol 0.02 to 20 $\mu\text{g/ml}$.

It is well known that in vitro and in vivo concentrations cannot be directly compared. The concentrations we used agree with those generally used in tissue culture (e.g., 3, 32, 33, 46). Different availability in vitro and in vivo might play a role. The test situation can be more adequately compared with the situation in the studies of oestrogen binding capacity in vitro. Two types of such binding have been demonstrated. One is found at physiological levels of the hormone - Jensen et al. (19) found it at 10^{-10} M. The receptors can then bind only a limited amount of the steroid, which is retained. The second type is found at much higher concentrations. The cells cannot be saturated and large amounts of steroids can be forced into the cells. These, however, do not retain the hormone. If these results are applied to the situation in the test systems used, it is possible that the cells are subject to an overwhelming pressure of steroids, which therefore enter irrespective of the presence of specific receptors.

What will be the effect on the living cell by the steroids? One quite likely effect would be toxic and inhibiting. Inhibiting effects were also seen in many experiments, especially at the highest concentrations. A close dose-dependent inhibition was also demonstrated (paper I, II) with all three steroids (testosterone, progesterone, 17- β -oestradiol) in most of the tumours tested, although some deviated from that pattern. When, as a control, corresponding studies were made with 17- α -oestradiol — which has a much lower biological activity — or with cholesterol, a much lower or no dose-dependent inhibition was seen (paper II, Fig. 4). This experiment suggests that the recorded inhibitions show some sort of specificity, as only the endocrinally active steroids exert it. It should also be stressed that — although mainly at the low concentrations used — stimulating effects were seen. These were more common in organ culture studies, but some were recorded also with the suspension method.

There are some studies on organ cultures of non-neoplastic human mammary gland tissue, where high concentrations of sex steroids were shown to have a positive influence. Cereani et al. (4) recorded maintenance and lobulo-alveolar development induced by progesterone at 1 $\mu\text{g/ml}$. Oestradiol at 0.001 $\mu\text{g/ml}$ increased lobulo-alveolar differentiation but 0.01 $\mu\text{g/ml}$ was toxic. Koyama et al. (22) recorded duct proliferation in rat mammary glands in organ culture with 17- β -oestradiol at concentrations up to 1 $\mu\text{g/ml}$. Progesterone at 1 $\mu\text{g/ml}$ induced "remarkable proliferative changes in the duct epithelium and a moderate development of lobular structures". This and similar findings support the idea that stimulative effects can be obtained with sex steroids at concentrations similar to those used in the present study. The effects seen might thus be more specific than could be imagined.

Tomkins (40) discussed two types of cell regulatory effects that can be obtained by various substances, sex steroids included. One

was called "specific" and is analogous with the specific retention of steroids after binding to specific receptors. The other was called "pleiotypic". It consists of a co-ordinated set of reactions: inhibition of RNA synthesis, protein synthesis, polysome formation glucose uptake, and nucleic acid precursor uptake, whereas protein degradation is stimulated. The inhibitory effects recorded in the present study could well be an expression of a "pleiotypic" response.

So far in the discussion, sarcoma cells have been regarded as non-target cells for sex steroids. Human sarcomas are at present not subject to sex steroid treatment. Starr (37) discussed the possible influence of growth hormone on sarcomas. Rat sarcomas can probably be arrested in their growth, but not in incidence, by oestrogen treatment (29). As far as I know, the possible presence of receptors for sex steroids on sarcoma cells has not been studied. The absence of such receptors in normal muscle cells (19) does not preclude the possibility that receptors could develop in malignant cells of mesodermal origin. Analogous phenomena have repeatedly been described, e.g., hormone production in certain lung cancers and appearance of fetal antigens on malignant cells, and are usually explained as the result of derepression of the genome in connexion with malignization or tumour progression. A further possibility is that sarcoma cells can metabolize sex steroids to other steroids for which receptors may be present: e.g., receptors for glucocorticoids exist in fibroblasts (21). As mentioned in Chapter 2, neoplastic mammary tissue can metabolize steroids to other active forms.

GENERAL SUMMARY

The present work deals with two different in vitro methods; one cell suspension, short term incubation method; and one organ culture method with incubation times up to 72 hours. With these methods, effects of sex steroids were measured as changes in DNA synthesis. Human mammary carcinomas and DMBA-induced rat mammary tumours were studied, human sarcomas and chemically- or virus-induced rat sarcomas being used as controls.

Effects, both stimulations and inhibitions, were found in both mammary tumours and sarcomas from the two species. This led to the investigation of differences between the two methods. The cell suspension method was less sensitive for detecting stimulations than the organ culture method. It also tended to overestimate the number of inhibitory effects. With the organ culture method, culture time was found to be important for the type of hormonal response recorded: a given tumour could yield widely different responses after different times of culture. Other possible sources of variation that could influence the results were investigated. With the organ culture technique, an interexperimental variation was found which — although small — might influence the effects.

The sex steroid concentrations used for in vitro cultures are discussed in the light of specific steroid binding of target tissue in vitro.

The work questions the specificity of recorded hormone effects of in vitro systems, at least measured as effects on DNA synthesis.

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